Remarks/Arguments

Claims 1-18 are pending in the application. Claims 16-18 are newly added. Claims 1-9 and 15 have been withdrawn from consideration pursuant to a lack of unity objection. Claims 10-14 and 16-18 are under consideration.

Response to Section 112, 1st and 2nd paragraph Rejections

Claims 10, 11, 13 and 14 have been rejected as indefinite. Claims 10-14 have been rejected for lack of enabling disclosure in the specification. Reconsideration is requested in view of the above claim amendments and the following remarks.

Claim 10 has been rejected as indefinite due to the presence of the phrase "extracellular pathogenic organism". The terminology no longer appears in the claims. The corresponding element now appearing in claim 10 is "pathogenic organism selected from the group consisting of bacteria, protozoa and fungus". Support is found at page 7, lines 11-14.

Claim 10 has been rejected as indefinite due to the terminology "immunogenic determinant". Examiner has proposed alternative language, which has been adopted.

Claim 11 has been rejected as indefinite because it is unclear what is encompassed by "stress-inducing stimuli" and "stress protein/antigenic peptide fragment complexes". Claim 11 has been amended to refer to heat shock as the stimuli, and to refer to the complexes as "heat shock protein/antigenic peptide fragment complexes". Furthermore, the final step of the claim now conforms to the objective of the preamble.

Claim 13 has been rejected due to the language "the composition is an aqueous composition". The claim has been rewritten to indicate that the composition comprises an aqueous carrier. Support is found at page 10, line 26.

Claim 14 has been rejected as indefinite because the condition being treated is not stated. Claim 14 has been rewritten as a method for vaccination against infection by a pathogenic organism, selected from bacteria, protozoa and fungus. It is respectfully submitted that the amendment overcomes the ground of rejection.

Claims 10-14 have been rejected for alleged lack of enabling disclosure. Examiner acknowledges that the instant specification adequately describes and provides supporting experimental methods and results exemplifying vaccines comprising one or more complexes between a heat shock protein (HSP) and an antigenic peptide fragment derived from the heat treatment of bacteria, but not other organisms. Submitted herewith are two papers, Morimoto *et al.*, *J. Biol. Chem.*, 67(31):21987-21990 (1992) and Bardwell *et al.*, *Proc Natl. Acad. Sci. USA* 81:848-852 (1984), describing the high degree of evolutionary conservation among the heat shock proteins across species. According to Morimoto *et al.*, genes for heat shock proteins are "among the most highly conserved with representatives from distinct prokaryotic and eukaryotic species having at least 50% identity". *Id.* at 21987. The high degree of conservation is exemplified by the 48% amino acid sequence identity in the proteins encoded by the *HSP70* gene of *Drosophila* and the homologous *dnaK* gene in *E. coli*. Bardwell *et al.* at 848.

One of ordinary skill in the art would not view the scope of the invention as limited to utilization of heat shock/antigenic peptide fragment complexes from bacteria. While bacterial infection may differ in certain respects from infections caused by fungi and protozoa, the difference is not relevant to the formation of the complexes, since the complexes are formed upon heat stressing of the infectious organism *per se*, not the infected host cell. The recognized high degree of evolutionary homolog among the heat shock proteins indicates that the same type of heat shock/antigenic peptide fragment complexes generated in bacteria, useful as vaccines against bacterial infection, would also form in the cells of the other pathogens, and provide useful vaccines against fungal and protozoal infection. It is thus respectfully submitted that the invention defined in claims 10-14, as amended, is adequately supported by the specification.

New claims 16, 17 and 18, depend from claims 10, 11 and 14, respectively, and are directed to embodiments of the invention wherein the complexes are formed by the heat stressing of bacteria. These claims are therefore believed free of the Section 112 enablement rejection.

Response to Section 102 Rejection

Laminet et al.

Claims 10, 11 and 13 have been rejected as allegedly anticipated by Laminet et al. Examiner states that a heat shock protein, by definition, is a protein produced in response to stress such as heat. However, this understanding of the function and the role of heat shock proteins within the cell is incorrect.

It is correct to say that heat shock proteins are upregulated following heat shocking. However, however they are also *constitutively expressed* in cells under normal cellular conditions, where they are involved with ongoing antigenic processing and presenting activities of the cell. Laminet does not teach heat-induced production of GroES or GroEL, but only constitutive expression. As seen in Example 3 of the instant application, constitutively expressed heat shock proteins bind proteins which are less immunogenic.

Laminet teaches neither the formation of a heat shock protein-antigenic peptide fragment complex, or the use of such a complex to induce an immune response.

Further, Examiner interprets the reference in Laminet to a GroEL/ES complex to mean a complex of a heat shock protein with an antigenic peptide. This understanding is also incorrect. The GroEL/ES complex as referenced in Laminet refers to the joining of the GroEL and GroES protein factors. Hence GroEL/ES does not form a heat shock protein complex in the same sense as the instant invention and as submitted by the Examiner on line 2, page 13 of the office action.

Laminet does not disclose a heat shock protein or other form of stress protein which is conjugated with an antigenic peptide, against which an immune response can be mediated. Claims 10, 11 and 13 are not anticipated by Laminet.

Srivastava (US Patent No 5,961,979)

Claims 10-14 have been rejected as allegedly anticipated by Srivastava. Srivastava relates to a stress protein-peptide complex for administration to a mammal which can induce a T cell immune response, this response inducing protection to a preselected *intracellular* pathogen. Examiner alleges that Srivastava's compositions anticipate the instant claims.

Srivastava provides "a stress protein-peptide complex *isolated from cells infected with* a preselected intracellular pathogen when administered to a mammal can effectively stimulate cellular immune responses against cells infected with the same pathogen" (column 4, line 56-60). The source and structure of the Srivastava complexes are totally distinct to those obtained according to the present invention. Specifically, in order to obtain the stress protein-peptide complexes required to perform the Srivastava invention, it is necessary to firstly select an intracellular pathogen against which you wish to induce immunity with the subsequently produced vaccine, and then *infect a eukaryotic cell with that preselected intracellular pathogen* (see column 9, lines 24-27). Further guidance is provided in Srivastava relating to the infection of eukaryotic cells with the preselected intracellular pathogen, starting at column 12, line 55 through column 13, line 49.

In Srivastava, the stress protein–peptide complexes which are used as the immunogenic determinant in a vaccine are comprised of stress proteins (heat shock proteins) which are derived from the *host eukaryotic cell* complexed to peptide *which is derived from the preselected intracellular pathogen* which is chosen to infect the eukaryotic cell.

The present invention results in the production of heat shock protein-peptide complexes which are fundamentally distinct to those produced in Srivastava. The heat shock protein-antigen complexes according to the claims of the present invention comprise heat shock protein which is derived from the *infectious agent itself*, which heat shock protein is coupled to an antigenic peptide *which is also derived from the infectious agent itself.* Unlike Srivastava, the infectious agents which form heat shock protein/antigen peptide complexes of the present invention are not infected into eukaryotic cells in order to extract complexes.

The present invention exemplifies, in Example 1, the fact that the infectious agents against which an immune response is desired are not put into eukaryotic cells, followed by heat-shocking of the eukaryotic cell. Rather, *it is the infectious agent itself which is heat shocked.* Accordingly, the heat shock protein-peptide complexes of the instant invention are both conceptually different and structurally distinct from the stress protein-peptide complexes of Srivastava.

At no point does Srivastava disclose or consider the disposal of the step of infecting a eukaryotic cell with the preselected infectious agent. Accordingly there is no consideration of the fact that a stress protein-peptide complex which is derived *directly* from the infectious agent may be of use in a vaccine to induce immunity against that pathogen.

The instant invention accordingly describes complexes which are not disclosed in Srivastava. Claims 10-14 are not anticipated by Srivastava.

Wallen et al. (US Patent No 5,747,332)

Claims 10, 11 and 13 have been rejected as allegedly anticipated by Wallen et al.

Wallen relates to methods for purifying and synthesizing heat shock protein complexes. The purification aspect of the Wallen invention relates to the use of a filtration column, such as an agarose gel column, which uses affinity binding to bind heat shock protein-peptide complexes.

In the second aspect of the Wallen invention, the filtration column can be used to form heat shock protein-peptide complexes by first binding heat shock protein complexes to the column and then running a peptide preparation through the column. The peptides apparently becoming associated with the bound heat shock protein molecules as they pass through the column.

The instant invention is directed to heat shock protein-peptide complexes which are formed following the heat treatment of infectious agents, such as bacteria. These complexes can then be formulated into a vaccine and administered to a mammal such as a human in order to induce immunity against the infectious agent. The complexes which result from the instant invention comprise a heat shock protein, which is derived from the infectious agent, and an antigenic peptide, which is also derived from the same infectious agent. The complex is formed *in-situ* within the infectious agent cell.

Wallen does not teach the specific combination of a heat shock protein and an associated peptide, and in particular does not each obtaining a heat shock protein and an associated peptide from the same cell type.

Although Wallen acknowledges that heat shock protein-peptide complexes can be used in vaccines, the instant invention makes the advance that a heat shock protein-peptide complex which is entirely derived from the same infectious agent can be used in a vaccine to induce immunity. Claims 10, 11 and 13 are therefore not anticipated by Wallen.

Hamel et al. (WO 96/40928)

Claims 10-14 have been rejected as allegedly anticipated by Hamel et al.

There are several reasons why the methods and teachings of Hamel would not inherently result in the heat shock protein complexes according to the claims of the instant invention. Having considered the experimental protocol which is taught by Hamel in relation to the purification steps which are performed in order to obtain the isolated HSPs, it would be clear to the person

skilled in the art that the conditions set forth for the isolation of the heat shock protein fragment from the cell supernatant uses 2% N-lauroyl sarcosine (see page 35, line 11). This chemical would result in the dissociation of any heat shock protein-peptide complexes which existed in the cellular preparations. Accordingly only heat shock proteins and not heat shock protein-peptide complexes would be provided in the cell supernatant of Hamel. No stress protein-peptide complexes would be isolated.

Further, Figure 2 of Hamel indicates that heat shock protein-peptide complexes are not present in the protein precipitate harvested from the centrifugation of the *S. pneumoniae* cells used in Hamel. Figure 2 shows a densitometric tracing showing profile of proteins from *S. pneumoniae* in the presence (- - -) or absence (-----) of exposure to heat shock. It is understood that the peaks shown for HSP 80, 72 and 62 represent these proteins without peptides attached (as is desired by the invention of Hamel). If peptides were complexed with the heat shock proteins, one would expect to see a further peak to the left of each of the peaks for HSP 80, 72 and 62. These additional peaks would represent the heat shock proteins with attached peptides. The complexing of the heat shock protein with antigenic peptides would result in the molecular weight of the complex being larger that the molecular weight of the heat shock protein alone. Since the presence of such peaks cannot be seen in Figure 2, it may be concluded that no heat shock protein-peptide complexes are present.

Furthermore, none of the examples provide in Hamel actually show the use of heat-induced heat shock proteins for conferring an immune response. All the appropriate examples relate to an embodiment of Hamel which utilizes recombinant heat shock proteins.

It is respectfully submitted that claims 10-14 are not anticipated by Hamel.

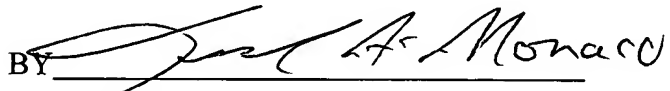
None of the asserted references anticipate the claims of the application. Reconsideration and withdrawal of the Section 102 rejection is respectfully submitted.

Conclusion

The claims of the application are believed in condition for allowance. An early action toward that end is earnest solicited.

Respectfully submitted

CAMILO ANTHONY LEO SELWYN COLACO

BY 

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Genetics

Major heat shock gene of *Drosophila* and the *Escherichia coli* heat-inducible *dnaK* gene are homologous

(DNA sequence comparison/gene evolution/archaebacteria)

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Communicated by James F. Crow, October 24, 1983

ABSTRACT **The *Escherichia coli dnaK* gene is homologous to the major heat shock-induced gene in *Drosophila* (*Hsp70*). The primary DNA sequence of the entire protein-coding region of the *dnaK* gene was determined and compared with that of the *Hsp70* gene of *Drosophila*. The two sequences are homologous; the *dnaK* gene could encode a 69,121-Da polypeptide, 48% identical to the hsp70 protein of *Drosophila*. The homology between the *Hsp70* gene of *Drosophila* and the *E. coli dnaK* gene illustrates the remarkable conservation of the heat shock genes in evolution. In contrast to *Drosophila* and *Saccharomyces cerevisiae*, both of which contain multigene families related to the *Hsp70* gene, hybridization analyses indicate that *E. coli* contains only a single *Hsp70*-related gene, *dnaK*. Hybridization between the DNA of an archaebacterium *Methanosarcina barkeri* and the *Hsp70* genes of *Drosophila*, *Saccharomyces*, and *E. coli* has been detected, suggesting the existence of *Hsp70*-related genes in the three "primary kingdoms": eukaryotes, eubacteria, and archaebacteria.**

When cells from a variety of species are quickly heated to a few degrees above their normal growth temperature, the synthesis of a small set of proteins is strongly and rapidly induced. The heat shock response has been most extensively studied in *Drosophila*, but a similar response has been observed in cells of a broad spectrum of eukaryotes: *Saccharomyces cerevisiae* (1), *Dictyostelium* (2), tobacco, hamsters, chickens, and humans (3, 4).

The function of the heat shock proteins is not known. However, the synthesis of heat shock proteins has been correlated with the acquisition of resistance to heat in eukaryotic cells (5). Agents that interfere with oxidative phosphorylation, as well as various chemicals such as ethanol, elicit the heat shock response (5, 6). Thus, the induction is thought to be a general response to stress, not merely a response to an alteration in temperature.

Evidence is accumulating that indicates that these induced proteins have been conserved throughout eukaryotic evolution. In many organisms, heat induces the synthesis of a protein of ≈70,000 Da. Antibodies raised to a 70,000-Da heat-shock protein (hsp70) from chicken crossreact with heat-shock proteins of similar molecular size in eukaryotic species as divergent as yeast and man (7). The predicted amino acid sequences of hsp70 proteins of yeast are 72% identical to the *Drosophila* hsp70 protein (ref. 8; unpublished observations).

A heat shock response has also been observed in a prokaryote, *Escherichia coli*. On temperature up-shift, the rate of synthesis of at least 14 polypeptides is enhanced. This induction is under the control of a gene called htp^R (9). Four of these HTP (*h*igh *t*emperature *p*roduction) proteins have been identified: the *groEL*, *groES*, and *dnaK* gene products, and a lysine tRNA synthetase. The first three proteins are essential for the growth of bacteriophage λ (10, 11, 18). Furthermore, $groEL^-$, $groES^-$, and $dnaK^-$ mutants that are temperature sensitive for bacterial growth at 43°C have been isolated (11, 12), suggesting that these genes are essential for cell viability. In this paper, we report that the *dnaK* gene of *E. coli* is homologous to the *Hsp70* heat shock genes of higher organisms, thus establishing a relationship between the prokaryotic and eukaryotic heat shock systems.

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MATERIALS AND METHODS

Bacteriophage and Plasmids. λdnaK, a phage capable of tranducing *E. coli* $dnaK^-$ to $dnaK^+$, and λdnaJdnaK phage were obtained from M. Feiss (13). Deletion derivatives of λdnaJdnaK were obtained from H. Uchida (14). Three plasmids containing yeast genes related to the *Drosophila Hsp70* gene were used. *YG100* (8) and *YG102* (15) are 97% identical to each other and 67% identical to *Drosophila Hsp70*. Transcription of these three genes is enhanced by heat shock. *YG101* is 67% related to *YG100* and *YG102*, and 56% identical to *Drosophila Hsp70*. Another plasmid, B8, contains a single copy of a 87C *Drosophila Hsp70* gene (16). In all cases pBR322 is the vector.

General Methods. Gel electrophoresis, blotting of DNA to nitrocellulose, nick-translation, and DNA sequence analysis were carried out as described (16, 17). Labeling of DNA by polynucleotide kinase was as described (16) except that prior to labeling, blunt or recessed 5′ ends of DNA were converted to protruding 5′ ends using DNA polymerase Klenow fragment (New England BioLabs). Five units of enzyme were incubated with 20–30 μg of digested DNA in 50 mM NaCl/7 mM $MgCl_2$/7 mM Tris·HCl, pH 7.4, for 30 min at room temperature (Z. Burton, personal communication).

In calculating the percentage amino acid identity, perfect amino acid matches were counted and divided by the sum of the total number of residues and the number of gapped residues. To calculate percentage nucleotide identity, matching bases were counted and divided by the sum of the total number of base pairs and the number of gaps.

DNA·DNA Hybridizations. Hybridization of DNA labeled by nick-translation to DNA bound to nitrocellulose filters was carried out under nonstringent conditions. The hybridization solution was 30% formamide/0.75 M NaCl/0.075 M sodium citrate/0.1% $NaDodSO_4$/1.0 mM EDTA/10 mM Tris·HCl, pH 7.5/1× Denhart's solution (8). The filters were incubated with probe overnight at 37°C after a 4-hr incubation in hybridization buffer in the absence of probe. The filters were then washed in 0.75 M NaCl/0.075 M sodium citrate/0.2% $NaDodSO_4$ at 37°C for 4 hr with three changes of the wash solution.

RESULTS

Hybridization of Eukaryotic *Hsp70* Genes to the *E. coli dnaK* Gene. To determine whether *E. coli* contains se-

Abbreviation: kb, kilobase(s).

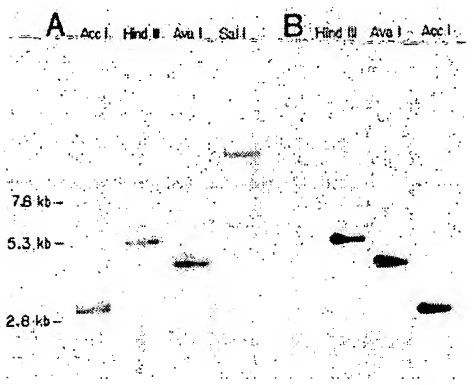

FIG. 1. Hybridization of a yeast heat shock gene and the *dnaK* gene to *E. coli* genomic DNA. Three-microgram portions of *E. coli* DNA were digested with restriction enzymes, electrophoresed on a 0.7% agarose gel, and transferred to nitrocellulose. (*A*) A fragment of a yeast *Hsp70* gene, *YG102*, encompassing the codons of amino acids 118–639 was labeled with ^{32}P by nick-translation and hybridized to the filter under low stringency. (*B*) A fragment of the *dnaK* gene encompassing the codons of amino acids 174–412 was labeled with ^{32}P by nick-translation and hybridized to the filter under conditions of low stringency.

quences related to eukaryotic 70,000-Da heat shock genes, hybridization experiments were carried out. *E. coli* genomic DNA was digested with restriction enzymes, subjected to gel electrophoresis, and transferred to nitrocellulose. The DNA was hybridized to a protein-coding portion of the yeast gene, *YG102*, which is related to the *Drosophila* 70,000-Da heat-shock gene. As shown in Fig. 1*A*, a single band of hybridization was observed after independent digestion with four different restriction enzymes. DNA of λ transducing phage containing the *dnaK* and *dnaJ* genes was fixed to nitrocellulose and probed with a portion of the protein-coding region of an *Hsp70*-related gene from yeast (*YG101*). *dnaK* was tested because it is a major heat shock gene in *E. coli* (9). An intense hybridization signal was observed, indicating probable similarity between the *dnaJ dnaK* region and the yeast gene. To map the region of hybridization on these phage, a yeast heat shock gene probe was hybridized to a series of λdnaJdnaK deletion derivatives. The *dnaK* gene had been previously mapped between the left end of the *Hin*dIII insert

```
                10         20         30         40         50
   1  ATGGGTAAAA TAATTGGTAT CGACCTGGGT ACTACCAACT CTTGTGTAGC
  51  GATTATGGAT GGCACCACTC CTCGCGTGCT GGAGAACGCC GAAGGCGATC
 101  GCACCACGCC TTCTATCATT GCCTATACCC AGGATGGTGA AACTCTAGTT
 151  GGTCAGCCGG CTAAACGTCA GGCAGTGACG AACCCGCAAA ACACTCTGTT
 201  TGCGATTAAA CGCCTGATTG GTCGCCGCTT CCAGGACGAA GAAGTACAGC

 251  GTGATGTTTC CATCATGCCG TTCAAAATTA TTGCTGCTGA TAACGGCGAC
 301  GCATGGGTCG AAGTTAAAGG CCAGAAAATG GCACCGCCGC AGATTTCTGC
 351  TGAAGTGCTG AAAAAAATGA AGAAAACCGC TGAAGATTAC CTGGGTGAAC
 401  CGGTAACTGA AGCTGTTATC ACCGTACCGG CATACTTTAA CGATGCTCAG
 451  CGTCAGGCAA CCAAAGACGC AGGCCGTATC GCTGGTCTGG AAGTAAAACG

 501  TATCATCAAC GAACCGACCG CAGCTGCGCT GGCTTACGGT CTGGACAAAG
 551  GCACTGGCAA CCGTACTATC GCGGTTTATG ACCTGGGTGG TGGTACTTTC
 601  GATATTTCTA TTATCGAAAT CGACGAAGTT GACGGCGAAA AAACCTTCGA
 651  AGTTCTGGCA ACCAACGGTG ATACCCACCT GGGGGGTGAA GACTTCGACA
 701  GCCGTCTGAT CAACTATCTG GTTGAAGAAT TCAAGAAAGA TCAGGGCATT

 751  GACCTGCGCA ACGATCCGCT GGCAATGCAG CGCCTGAAAG AAGCGGCAGA
 801  AAAAGCGAAA ATCGAACTGT CTTCCGCTCA GCAGACCGAC GTTAACCTGC
 851  CATACATCAC TGCAGACGCG ACCGGTCCGA AACACATGAA CATCAAAGTG
 901  ACTCGTGCGA AACTGGAAAG CCTGGTTGAA GATCTGGTAA ACCGTTCCAT
 951  TGAGCCGCTG AAAGTTGCAC TGCAGGACGC TGGCCTGTCC GTATCTGATA

1001  TCGACGACGT TATCCTCGTT GGTGGTCAGA CTCGTATGCC AATGGTTCAG
1051  AAGAAAGTTG CTGAGTTCTT TGGTAAAGAG CCGCGTAAAG ACGTTAACCC
1101  GTACGAAGCT GTAGCAATCG GTGCTGCTGT TCAGGGTGGT GTTCTGACTG
1151  GTGACGTAAA AGACGTACTG CTGCTGGACG TTACCCCGCT GTCTCTGGGT
1201  ATCGAAACCA TGGGCGGTGT GATGACGACG CTGATCGCGA AAAACACCAC

1251  TATCCCGACC AAGCACAGCC AGGTGTTCTC TACCGCTGAA GACAACCAGT
1301  CTGCGGTAAC CATCCATGTG CTGCAGGGTG AACGTAAACG TGCGGCTGAT
1351  AACAAATCTC TGGGTCAGTT CAACCTAGAT GGTATCAACC CGGCACCGCG
1401  CGGCATGCCG CAGATCGAAG TTACCTTCGA TATCGATGCT GACGGTATCC
1451  TGCACGTTTC CGCGAAAGAT AAAAACAGCG GTAAAGAGCA GAAGATCACC

1501  ATCAAGGCTT CTTCTGGTCT GAACGAAGAT GAAATCCAGA AAATGGTACG
1551  CGACGCAGAA GCTAACGCCG AAGCTGACCG TAAGTTTGAA GAGCTGGTAC
1601  AGACTCGCAA CCAGGGCGAC CATCTGCTGC ACAGCACCCG TAAGCAGGTT
1651  GAAGAAGCAG GCGACAAACT GCGGGCTGAC GACAAAACTG CTATCGAGTC
1701  TGCGCTGACT GCACTGGAAA CTGCTCTGAA AGGTGAAGAC AAAGCCGCTA

1751  TCGAAGCGAA AATGCAGGAA CTGGCACAGG TTTCCCAGAA ACTGATGGAA
1801  ATCGCCCAGC AGCAACATGC CCAGCAGCAG ACTGCCGGTG CTGATGCTTC
1851  TGCAAACAAC GCGAAAGATG ACGATGTTGT CGACGCTGAA TTTGAAGAAG
1901  TCAAAGACAA AAAATAA
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FIG. 3. Nucleotide sequence of the protein-coding portion of the *dnaK* gene. All of the sequence was determined on both strands of the DNA with the exception of a 70-base pair stretch from nucleotide 370–440, which was determined twice on one strand. Two independent clones of *dnaK* were sequenced over the entire length to guard against errors due to cloning artifacts. We have sequenced across all restriction sites, so we are certain no gaps exist in our sequence.

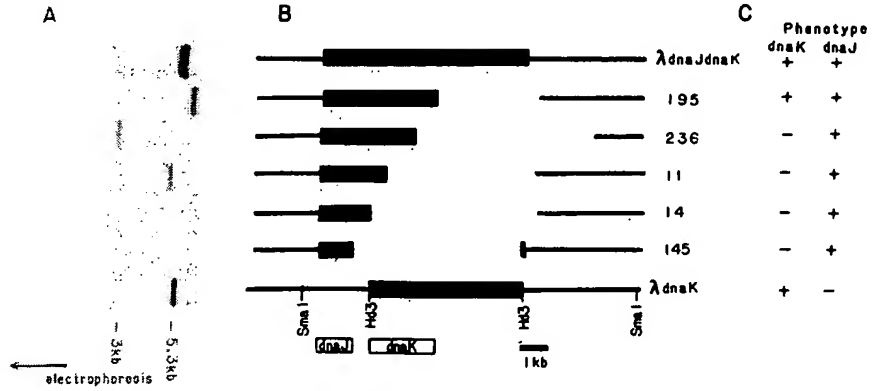

FIG. 2. Mapping of the hybridization between *E. coli* and eukaryotic heat shock genes to the *dnaK* gene. (*A*) DNA from λdnaK and deletion derivatives derived from λdnaJdnaK were cleaved with *Sma* I and *Hin*dIII (Hd3), electrophoresed through 0.7% agarose, and transferred to nitrocellulose. A fragment of the yeast heat shock gene *YG102* encompassing the codons of amino acids 118–639 was hybridized to the filter. (*B*) The physical structures of the λ deletions shown were determined by Saito (14) using heteroduplex analysis. *E. coli* DNA is represented by the thick lines. Thin lines represent flanking phage vector. (*C*) The dnaK and dnaJ phenotypes of these phages were determined by Saito (14) by testing the ability of each of these deletion mutants to plaque on bacterial strains containing mutations in the *dnaK* and *dnaJ* genes. The limits of *dnaJ* and *dnaK* designated at the bottom of the figure are based on such tests of these deletion derivatives.

in λ*dnaK* and the end point of the deletion in λdnaJ-dnaKΔ195 (ref. 14; Fig. 2*B*) by testing the ability of the deletions to complement *dnaK*⁻ mutations. DNA from these phages were cleaved with restriction enzymes that excise the bacterial DNA from phage DNA and also separate the *dnaK* gene from the *dnaJ* gene. When the DNA was hybridized to a yeast heat shock gene probe, a band of hybridization was observed only with those deletion derivatives that contained DNA from the *dnaK* gene region (Fig. 2*A*). The only fragments that hybridized were those containing the *dnaK* sequences. A labeled plasmid (B8) containing a copy of the *Drosophila Hsp70* gene was also hybridized to a similar blot of the λdnaJdnaK deletions, and it showed a similar pattern of hybridization (results not shown).

DNA Sequence Analysis of *dnaK* and Comparison to *Hsp70* Gene. The 5.3-kilobase (kb) *Hin*dIII fragment containing sequences that hybridized to the *Drosophila* and yeast *Hsp70* genes was subcloned into pBR322. Restriction enzyme mapping and hybridization analyses localized the region of *Hsp70* hybridization to between an *Nru* I site in the middle of the insert and a *Hin*dIII site near the *dnaJ* gene.

The primary DNA sequence of this region (Fig. 3) was determined. A single long open-reading frame that could code for a protein of 69,121 Da was identified (Fig. 4). Both the predicted amino acid composition and the amino-terminal sequence agrees with that determined from the analysis of purified dnaK protein, except that the protein lacks an NH_2-terminal methionine (C. P. Georgopoulos, personal communication). This modification would decrease the size of the protein to 68,990 Da. A stop codon immediately precedes the AUG designated as the start codon, thus prohibiting initiation of *dnaK* at a methionine upstream. A comparison of the protein-coding region of the *dnaK* gene and the *Drosophila Hsp70* gene (16) reveals 57% identity at the nucleotide level and 48% identity at the amino acid level (Fig. 4). Some regions of the polypeptide show a greater similarity. Between amino acid 138 and amino acid 183, >90% of the amino acids are the same, and between amino acid 365 and 489, 64% are

```
E. coli dnaK  Met Gly Lys Ile Ile Gly Ile Asp Leu Gly Thr Thr Asn Ser Cys Val Ala Ile Met Asp Gly Thr Thr Pro Arg Val Leu Glu Asn Ala aa30
Dr. hsp70      *  Pro  Δ  Ala  *  *  *  *  *  *  *  *  Tyr  *  *  *  Gly Val Tyr Gln His Gly Lys Val Glu Ile Asn Ala Tyr Asp

E. coli dnaK  Glu Gly Asp Arg Thr Thr Pro Ser Ile Ile Ala Tyr Thr Gln Asp Gly Glu Thr Leu Val Gly Gln Pro Ala Lys Arg Gln Ala Val Thr aa60
Dr. hsp70      Gln  *  Asn  *  *  *  *  *  Tyr Val  *  Phe  *  Δ  *  Ser  *  Arg  *  Asn  *  Glu  *  *  *  Asn  *  Val Ala Met

E. coli dnaK  Asn Pro Gln Asn Thr Leu Phe Ala Ile Lys Arg Leu Ile Gly Arg Arg Phe Gln Asp Glu Glu Val Gln Arg Asp Val Ser Ile Met Pro aa90
Dr. hsp 70     *  *  Arg  *  *  Val  *  Asp Ala  *  *  *  *  *  *  Lys Tyr Asp  *  Pro Lys Ile Ala Glu  *  Met Lys His Trp  *

E. coli dnaK  Phe Lys Ile Ile Ala Ala Asp Asn Gly Asp Ala Trp  Δ  Val Glu Val Lys Gly Gln  Δ  Lys Met Ala Pro Pro  Δ  Gln Ile Ser Ala aa117
Dr. hsp70      *  *  Val Val Ser Asp Gly Gly Lys Pro Lys Ile Gly  *  *  Tyr  *  *  Glu Ser  *  Arg Phe Ala  *  Glu Glu  *  *  Ser

E. coli dnaK  Glu Val Leu Lys Lys Met Lys Lys Thr Ala Glu Asp Tyr Leu Gly Glu Pro Val Thr Glu Ala Val Ile Thr Val Pro Ala Tyr Phe Asn aa147
Dr. hsp70      Met  *  *  Thr  *  *  *  Glu  *  *  *  Ala  *  *  *  *  Ser Ile  *  Asp  *  *  *  *  *  *  *  *  *  *

E. coli dnaK  Asp Ala Gln Arg Gln Ala Thr Lys Asp Ala Gly Arg Ile Ala Gly Leu Glu Val Lys Arg Ile Ile Asn Glu Pro Thr Ala Ala Ala Leu aa177
Dr. hsp70      *  Ser  *  *  *  *  *  *  *  *  *  His  *  *  *  *  Asn  *  Leu  *  *  *  *  *  *  *  *  *  *  *

E. coli dnaK  Ala Tyr Gly Leu Asp Lys  Δ  Gly Thr Gly Asn Arg Thr Ile Ala Val Tyr Asp Leu Gly Gly Gly Thr Phe Asp Ile Ser Ile Ile Glu aa206
Dr. hsp70      *  *  *  *  *  *  Asn Leu Lys  *  Glu  *  Asn Val Leu Ile Phe  *  *  *  *  *  *  *  *  Val  *  *  Leu Thr

E. coli dnaK  Ile Asp Glu Val Asp Gly Glu Lys Thr Phe Glu Val Leu Ala Thr Asn Gly Asp Thr His Leu Gly Gly Glu Asp Phe Asp Ser Arg Leu aa236
Dr. hsp70      *  *  *  Gly  Δ  Δ  Δ  Ser Leu  *  *  *  Arg Ser  *  Ala  *  *  *  *  *  *  *  *  *  *  *  Asn  *  *

E. coli dnaK  Ile Asn Tyr Leu Val Glu Glu Phe Lys Lys Asp Gln Gly Ile Asp Leu Arg Asn Asp Pro Leu Ala Met Gln Arg Leu Lys Glu Ala Ala aa266
Dr. hsp70      Val Thr His  *  Ala  *  *  *  *  Arg Lys Tyr Lys Lys  *  *  *  Ser Asn  *  Arg  *  Leu Arg  *  *  Arg Thr  *  *

E. coli dnaK  Glu Lys Ala Lys Ile Glu Leu Ser Ser Ala Gln Gln Thr Asp Val Asn Leu Pro Tyr Ile Thr Ala Asp Ala Thr Gly Pro Lys His Met aa296
Dr. hsp70      *  Arg  *  *  Arg Thr  *  *  *  Ser Thr Glu Ala Thr Ile Glu  Δ  Δ  Δ  *  Asp  *  Leu Phe Glu  *  Δ  Gln Asp Phe

E. coli dnaK  Asn Ile Lys Val Thr Arg Ala Lys Leu Glu Ser Leu Val Glu Asp Leu Val Asn Arg Ser Ile Glu Pro Leu Lys Val Ala Leu Gln Asp aa326
Dr. hsp70      Tyr Thr  *  *  Ser  *  *  Arg Phe  *  Glu  *  Cys Ala Asn  *  Phe Arg Asn Thr Leu Gln  *  Val Glu Lys  *  *  Asn  *

E. coli dnaK  Ala Gly Leu Ser Val Ser Asp Ile Asp Asp Val Ile Leu Val Gly Gly Gln Thr Arg Met Pro Met Val Gln Lys Lys Val Ala Glu Phe aa356
Dr. hsp70      *  Lys Met Asp Lys Gly Gln  *  His  *  Ile Val  *  *  *  *  *  Ser  *  *  Ile  *  Lys  *  *  Ser Leu Leu Gln  *  *

E. coli dnaK  Phe  Δ  Gly Lys Glu Pro Arg Lys Asp Val Asn Pro Asp Glu Ala Val Ala Ile Gly Ala Ala Val Gln Gly Gly Val Leu Thr Gly Asp aa385
Dr. hsp70      *  His  *  *  Asn Leu Asn Leu Ser Ile  *  *  *  *  *  *  *  Tyr  *  *  *  *  *  Ala Ala Ile  *  Ser  *  *

E. coli dnaK   Δ  Δ  Δ  Δ  Val Lys Asp Val Leu Leu Leu Asp Val Thr Pro Leu Ser Leu Gly Ile Glu Thr Met Gly Gly Val Met Thr Thr Leu aa411
Dr. hsp70     Gln Ser Gly Lys Ile Gln  *  *  *  *  Val  *  *  Ala  *  *  *  *  *  *  *  *  *  Ala  *  *  *  *  *  Lys  *

E. coli dnaK  Ile Ala Lys Asn Thr Thr Ile Pro Thr Lys His Ser Gln Val Phe Ser Thr Ala Glu Asp Asn Gln Ser Ala Val Thr Ile His Val Leu aa441
Dr. hsp70      *  Glu Arg  *  Cys Arg  *  *  Cys  *  Gln Thr Lys Thr  *  *  *  Tyr Ser  *  *  *  Pro Gly  *  Ser  *  Gln  *  Tyr

E. coli dnaK  Gln Gly Glu Arg Lys Arg Ala Ala Asp Asn Lys Ser Leu Gly Gln Phe Asn Leu Asp Gly Ile Asn Pro Ala Pro Arg Gly Met Pro Gln aa471
Dr. hsp70      Glu  *  *  *  Ala Met Thr Lys  *  *  Asn Ala  *  *  Thr  *  Asp  *  Ser  *  *  Pro  *  *  *  *  *  Val  *  *

E. coli dnaK  Ile Glu Val Thr Phe Asp Ile Asp Ala Asp Gly Ile Leu His Val Ser Ala Lys Asp Lys Asn Ser Gly Lys Glu Gln Lys Ile Thr Ile aa501
Dr. hsp70      *  *  *  *  *  *  Leu  *  *  Asn  *  *  *  Asn  *  *  *  *  Glu Met Ser Thr  *  *  Ala Lys Asn  *  *  *

E. coli dnaK  Lys Ala Ser Ser Gly  Δ  Leu Asn Glu Asp Glu Ile Gln Lys Met Val Arg Asp Ala Glu Ala Asn Ala Glu Ala Asp Arg Lys Phe Glu aa530
Dr. hsp70      *  Asn Asp Lys  *  Arg  *  Ser Gln Ala  *  *  Asp Arg  *  *  Asn Glu  *  *  Lys Tyr  *  Asp Glu  *  Glu  *  His Arg

E. coli dnaK  Glu Leu Val Gln Thr Arg Asn Gln Gly Asp His Leu Leu His Ser Thr Arg Lys Gln Val Glu Glu Ala  Δ  Gly Asp Lys Leu Pro Ala aa559
Dr. hsp70     Gln Arg Ile Thr Ser  *  *  Ala Leu Glu Ser Tyr Val Phe Asn Val Lys Gln Ser  *  *  Gln  *  Pro Ala Gly  *  *  Asp Glu

E. coli dnaK  Asp Asp Lys Thr Ala Ile Glu Ser Ala Leu Thr Ala Leu Glu Thr Ala Leu Lys Gly Glu Asp Lys Ala Ala Ile Glu  Δ  Δ  Δ  Ala aa586
Dr. hsp70      Ala  *  *  Asn Ser Val Leu Asp Lys Cys Asn Glu Thr Ile Arg Trp  *  Asp Ser Asn Thr Thr  *  Glu Lys  *  Glu Phe Asp His

E. coli dnaK  Lys Met Gln Glu Leu Ala Gln Val Ser Gln Lys Leu Met Glu Ile Ala Gln Gln Gln His Ala Gln Gln Gln Thr Ala  Δ  Gly Ala  Δ  aa614
Dr. hsp70      *  *  Glu  *  *  Δ  Δ  Δ  Thr Arg His Cys Ser Pro  *  Met Thr Lys Met  *  Gln  *  Gly Ala Gly  *  Ala  *  Gly Pro

E. coli dnaK   Δ  Asp Ala Ser Ala Asn Asn Ala Lys Asp Asp Asp Val Val Asp Ala Glu  Δ  Δ  Δ  Phe Glu Glu Val Lys Asp Lys Lys END
Dr. hsp70     Gly Ala Asn Cys Gly Gln Gln  *  Gly Gly  Δ  Δ  Phe Gly Gly Tyr Ser Gly Pro Thr Val  *  *  *  Asp  Δ  Δ  Δ  *
```

FIG. 4. Comparison of the predicted amino acid sequence of *E. coli* dnaK protein and *Drosophila* (Dr.) hsp70. Those amino acids identical in both sequences are shown by an asterisk. The first amino acid (aa) of the dnaK protein is designated 1. Those amino acids deleted in one sequence relative to the other are shown by a triangle.

the same. If one divided the dnaK protein into five equal segments, the second and fourth are more conserved (66% and 58%) than the first, third, and fifth (46, 42, and 23% respectively). *dnaK* is also homologous to the yeast *Hsp70*-related genes used as hybridization probes. At the nucleotide level, *dnaK* is 57% identical to two yeast genes, *YG101* and *YG102*. The amino acid residues conserved from *E. coli* to *Drosophila* are nearly all conserved to yeast as well. These regions of high identity may represent functionally conserved regions of the protein.

Hybridization of Isolated *dnaK* to *E. coli* Genomic DNA. Since it has been shown that the *S. cerevisiae* and *Drosophila melanogaster* genomes contain a family of *Hsp70*-related genes (8, 19), we wanted to determine whether *E. coli* also contained multiple *Hsp70*-related genes. The *dnaK* gene was hybridized under nonstringent conditions to fractionated *E. coli* genomic DNA. A single band was observed after hybridization to genomic DNA digested independently with three different restriction enzymes (Fig. 1*B*). Under similar hybridization conditions a yeast *Hsp70* fragment hybridized to yeast genomic DNA reveals 8–10 *Hsp70* homologous genes (8). The detection of only one band indicates that the *E. coli* genome contains a single *Hsp70* homologous sequence—*dnaK*.

Hybridization of 70,000-Da Heat-Shock Genes to an Archaebacterium, *Methanosarcina barkeri*. In an attempt to further establish the conservation of *Hsp70* genes in evolution, *M. barkeri* genomic DNA was digested with restriction enzymes, blotted to nitrocellulose, and hybridized to protein-coding portions of *Hsp70*-related genes. Three probes, one from *E. coli dnaK*, one from yeast, and one from *Drosophila Hsp70* were used in separate hybridizations. The same single band of hybridization was observed using either yeast and *Drosophila* or *E. coli* and *Drosophila* DNA as probe (Fig. 5). We suggest that *M. barkeri* contains *Hsp70*-related sequences.

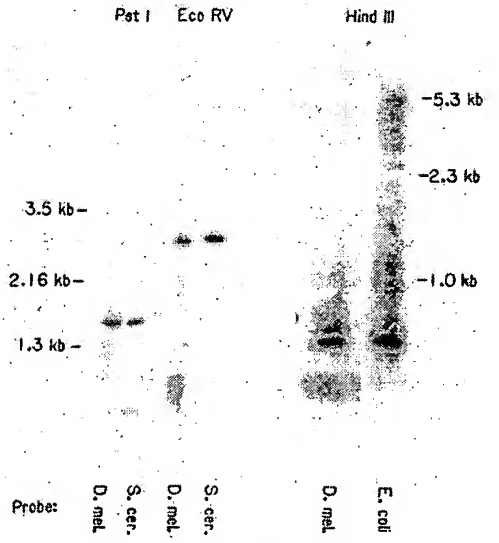

FIG. 5. Hybridization of *Hsp70* genes to DNA of an archaebacterium. *M. barkeri* genomic DNA was digested with restriction enzymes, electrophoresed, and transferred to nitrocellulose. Three protein-coding fragments of *Hsp70*-related genes were used as hybridization probes: a fragment of the *E. coli dnaK* gene encompassing amino acids 23–325, a fragment of the yeast (S. cer.) *YG102* gene encompassing amino acids 118–639, and a fragment of the *Drosophila* (D. mel.) *Hsp70* gene encompassing amino acids 18–337.

DISCUSSION

Because 70,000-Da heat shock-inducible genes exist in two distantly related species, *D. melanogaster* and *S. cerevisiae*, we wondered whether *E. coli* contained *Hsp70*-related sequences. A heat-inducible gene in *E. coli*, *dnaK*, is 57% identical to the major heat shock gene of *Drosophila* (*Hsp70*). This identity extends over the entire protein-coding region with some regions showing extreme conservation. A comparison of the predicted amino acid sequences of the *dnaK* and *Drosophila Hsp70* genes showed 48% overall identity. This identity increased to >90% in one 46-amino acid segment. The *dnaK* gene is also homologous to a yeast *Hsp70* gene. The sequence of the yeast gene is 57% identical to *dnaK* and 67% identical to the *Drosophila Hsp70*. Given the high degree of similarity observed between *dnaK* and other 70,000-Da heat shock genes, we assert that these genes have diverged from a common ancestor rather than converged from unrelated sequences. These results are a remarkable demonstration of prokaryotic–eukaryotic sequence homology.

Based on comparison of 5S RNA sequences, Hori and Osawa (20) have estimated that the prokaryotes and eukaryotes diverged about 1.8×10^9 years ago. Only a very few proteins have maintained recognizable sequence similarity between prokaryotic and eukaryotic species (21, 22). *c*-type cytochromes, serine proteases, and the β-subunit of the ATP synthetase complex are among those that have been well conserved. Cytochrome *c* shows 10–48% similarity between eukaryotic and prokaryotic species. Bacterial trypsin from *Streptomyces griseus* is 26–31% identical to eukaryotic serine proteases. One very highly conserved protein is the β-subunit of ATP synthetase (23). Sequences from maize chloroplasts and beef mitochondria are 64% and 69% identical to the *E. coli* β-subunit. The homology between the dnaK protein and *Drosophila* hsp70 is thus comparable with the homology seen between some of the most highly conserved proteins known.

The archaebacteria composed of the methanogens, extreme halophiles, and acidophiles form a unique biological grouping phylogenetically distant from typical bacteria (eubacteria) and eukaryotes. Three primary kingdoms have been proposed (24)—the eukaryotes, the eubacteria, and the archaebacteria. The archaebacteria differ significantly from eubacteria and eukaryotes. For example, all eubacteria contain muramic acid in their cell walls while archaebacteria do not. Membranes of archaebacteria contain unusual lipids not found in members of the two other groups and tRNA base modifications commonly found in eukaryotic and prokaryotic tRNA are absent in archaebacteria. The two bacterial lines of descent appear to be no more related to one another than either of them is to the eukaryotes (24). The detection of *Hsp70*-related sequences in an archaebacteria thus dramatically shows the extreme conservation of the *Hsp70* gene throughout evolution.

Both the *Drosophila* and yeast genomes contain a family of *Hsp70*-related genes (8, 19). In both cases, some members are heat shock inducible while others are transcribed under normal growth conditions and not inducible by heat treatment. Evidence presented here indicates that there is only one *Hsp70*-related gene, *dnaK*, in the *E. coli* genome. However, under normal growth conditions at 37°C the dnaK protein accounts for 1.4% of the weight fraction of cellular protein and is the seventh most abundant protein in the cell (25). Transcripts of one of the *Drosophila* Hsp70-related proteins (Hsc4) are very abundant in cells in all stages of development, comparable in abundance to some actin mRNAs (26). Therefore, in both eukaryotes and prokaryotes Hsp70-related proteins appear to be abundant under normal physiological conditions.

Although the function of the heat shock proteins is not known, the information from eukaryotic species including the abundance of these proteins suggests structural roles for the heat shock proteins either in the nucleo- or cytoskeleton. Studies of avian and mammalian heat shock proteins have shown that hsp70 is identical to a highly conserved polypeptide previously shown to copurify with intermediate filaments and microtubules (3). Antibodies to chicken hsp70 stains a pattern of stress fibers in the cytoplasm (27). Unfortunately, the information available concerning the dnaK protein does not obviously suggest its function in cellular metabolism. The dnaK protein is required for the replication of the phages λ and P22 but not T4, T7, φX174, or fd (28). Mutant λ phage able to grow on a *dnaK*⁻ strain contain an altered *P* gene product (10). The *P* gene product is thought to interact with the λ *O* gene product and the host *dnaB* gene product to form a replisome structure at λ *ori* (29). The temperature-sensitive phenotype of some *dnaK* mutants is strong evidence that the *dnaK* gene is required for the normal growth of *E. coli*. It has been suggested that *dnaK* is involved in host DNA replication (30). However, the existence of mutants that prevent λ DNA replication but do not affect host growth (30) suggests that the functions involved in host and phage metabolism may be different. Further genetic and biochemical analysis of *dnaK* and its protein product should help elucidate the role of *hsp70* in both normal and stress situations.

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Minireview

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Transcriptional Regulation of Heat Shock Genes

A PARADIGM FOR INDUCIBLE GENOMIC RESPONSES

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The heat shock response offers an ideal paradigm to understand how the cell recognizes and responds to acute and chronic exposures to environmental and physiological stress. Of the numerous inducible genomic responses, the heat shock response has contributed fascinating insights into the molecular and cellular mechanisms of adaptation, ranging from the regulation of heat shock gene expression to the function of stress proteins. The recent cloning of multiple heat shock transcription factor (HSF) genes in higher eukaryotes and studies on the biochemical and cellular properties of HSFs have revealed several novel features of the transcriptional response.

Common to all organisms is an essential, highly conserved, and exquisitely regulated cellular response to suboptimal physiological conditions. The activation of stress gene expression resulting in the elevated synthesis of a family of stress-induced or heat shock proteins (hsp)[1] ensures survival under stressful conditions, which, if left unchecked, leads to irreversible cell damage and ultimately cell death. The stress-induced proteins, some of which are also known as molecular chaperones, have essential roles in protein biosynthesis, specifically in the transport, translocation, and folding of proteins.

The genes encoding heat shock proteins are among the most highly conserved with representatives from distant prokaryotic and eukaryotic species having at least 50% identity (1, 2). Following heat shock, or exposure to other forms of stress, the synthesis of a small number of proteins dramatically increases; these are known as the "classical" heat shock proteins of molecular size 104, 90, 70, 60, and 20 kDa (3–6). The majority of studies have concentrated on the eukaryotic 70-kDa protein heat shock genes, which are ancestrally related to *Escherichia coli* dnaK, and encode a large multigene family of proteins including: 1) the constitutively expressed and primarily cytoplasmic hsc70 (p72), 2) mitochondrial p75, 3) lumen-localized and glucose-responsive grp78/BiP, 4) the inducible hsp72, and 5) the major constitutive and inducible hsp70 (7–18).

Among the puzzling aspects of the heat shock response has been the diversity of chemicals and physiological conditions that induce heat shock protein synthesis. Although the term "heat shock" has restrictive connotations, it does provide a useful historical reference. As the diversity of conditions that results in the elevated synthesis of heat shock proteins has expanded, the paradigm has become more generally known as the "stress response." The list of conditions known to induce hsp70 gene expression can be subdivided to three broad categories. As shown in Fig. 1, they include: 1) environmental stresses including exposure to heat shock, amino acid analogues, and heavy metals; 2) non-stress conditions including normal cell growth, development, differentiation, and activation by certain oncogenes; and 3) pathophysiology and disease states. Given the diversity of these cellular responses, a major goal is to understand the mechanism(s) by which physiological stress is detected and quantified and how this information is transduced to the transcriptional apparatus. A number of questions have emerged. For example, what is the receptor for physiological stress, and how does it sense stress at the molecular level?

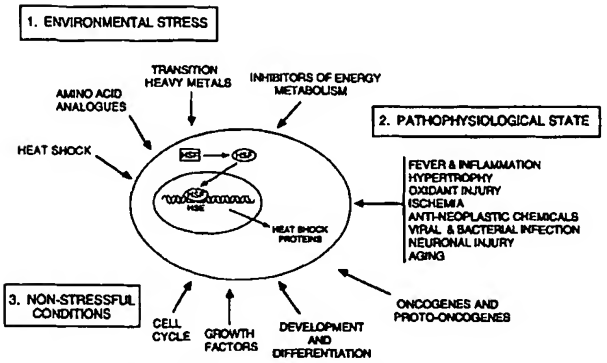

FIG 1. **Inducers of the cellular stress response.** Representation of three general classes of conditions known to result in the elevated expression of stress proteins including: *1*, environmental/physiological stress; *2*, pathophysiological states including conditions of disease; and *3*, non-stressful conditions such as cell growth and development. Each condition acts on the cell as diagrammed in this figure, and in the case of environmental stress and certain pathophysiological states leads to the activation of HSF, which enters the nucleus and binds to HSE in the promoters of heat shock genes. This leads to the activation of heat shock gene expression and the synthesis of heat shock proteins. Not shown here but discussed in the text are the non-HSE-mediated conditions for activation of heat shock genes.

This review will examine the mechanism of heat shock gene transcriptional induction in eukaryotic cells from the view of the hsp70 gene. Other perspectives on the regulation, expression, and function of heat shock proteins are available in recent reviews and monographs (19–28).

General Features of hsp70 Heat Shock Gene Transcription

The hsp70 gene has been well studied as a classical heat shock-responsive gene (29, 30). In addition the human hsp70 gene is expressed at the G1/S boundary of the cell cycle, in response to certain growth factors, hormones, mitogens, serum stimulation, and viral infection (26, 31–34). The promoter region of the human hsp70 gene reflects this complexity and contains multiple heat shock elements (HSE: inverted repeats of the pentameric sequence nGAAn), which confer stress inducibility and a basal promoter region comprised of a complex array of cis-binding sites (Sp1, CCAAT, ATF) (35–43). These multiple cis-regulatory elements confer a range of constitutive and inducible transcriptional responses to the hsp70 gene and are common to the promoters of other heat shock genes (44–46).

One of the best studied aspects of heat shock gene expression

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[1] The abbreviations used are: hsp, heat shock protein(s); grp, glucose-regulated protein; BiP, immunoglobulin binding protein; ATF, activating transcription factor; HSF, heat shock transcription factor; HSE, heat shock element(s); E1a13S, adenovirus early region 1a 13 S mRNA-encoded protein; aa, amino acid(s); Sp1, simian protein-1.

is the stress-induced transcriptional response. In eukaryotes, the increased transcription of heat shock genes is mediated by the activation of HSF (48–55). In the yeast *Saccharomyces cerevisiae*, HSF constitutively binds DNA and undergoes heat-induced phosphorylation with an increase in transcriptional activity. In higher eukaryotes, HSF is maintained in a non-DNA binding state in unstressed cells and in response to heat shock acquires an oligomeric state and binds to the HSE in the promoters of all heat shock-responsive genes (47, 55–61, 69). The response to heat shock is rapid; the activation of the DNA binding form of HSF can be detected within minutes of temperature elevation. The kinetics and magnitude of DNA binding activity during heat shock are proportional to the transcriptional response; however, HSF DNA binding activity does not always correlate with transcriptional activity (56, 61, 62). In mammalian and invertebrate cells, activation of HSF involves a number of inducible events including oligomerization, acquisition of DNA binding, translocation into the nucleus, and increase in transcriptional activity (58, 59, 63).[2] In addition, HSF isolated from heat-shocked yeast and human cells is phosphorylated (65–67).[2] However, phosphorylation is not essential for all of the properties of activated HSF. Analysis of HSF activated in mammalian cells shows that treatment with heat or cadmium sulfate results in the maximally phosphorylated state while amino acid analogue (azetidine)-induced HSF exhibits all of the features of the heat-induced HSF yet does not acquire additional phosphorylation.[2] These results reveal that phosphorylation of HSF is not essential for oligomerization, acquisition of DNA binding activity, or translocation of HSF into the nucleus. However, phosphorylation may be important for attaining maximal inducible transcriptional activity or for attenuation of the heat shock response. The heat shock transcriptional response is reversible at intermediate heat shock temperatures or upon return to control temperatures; likewise HSF undergoes conversion back to its control non-DNA binding state. In contrast, exposure to extreme temperatures results in sustained heat shock gene transcription and HSF DNA binding activity (61, 62, 67, 68).[2]

Heat Shock Factor Genes: Evidence for Distinct Functional Roles

HSF genes have been cloned from a number of organisms including yeasts, insect, tomato, chicken, mouse, and humans. Because HSF is encoded by a single gene in the yeasts *S. cerevisiae* and *Kluyveromyces lactis* and in the invertebrate *Drosophila* (57, 63, 66, 70) it was surprising when three HSFs were identified in tomato and chicken and at least two HSFs in human and mouse cells (71–74).[3] The cloned HSFs vary in size, from 301 amino acids (aa) for tomato HSF24, 512 aa for tomato HSF8, 491, 503, and 529 aa for chicken, mouse, and human HSF1, 564, 517, and 536 aa for chicken, mouse, and human HSF2, 467 aa for chicken HSF3, 691 aa for *Drosophila* HSF, and 833 aa for *S. cerevisiae*. Despite this striking variation in size and overall homology of less than 40%, all HSFs have two highly conserved features: the amino-terminal localized DNA binding domain of approximately 100 amino acids and a motif of hydrophobic heptad repeats, which mediates the oligomerization of HSF (55, 57, 59, 63). The level of conservation in the DNA binding domain is not surprising as each HSF binds to the highly conserved heat shock element DNA binding motif. By deletion analysis, the heptad hydrophobic amino acid repeats have been shown to be sufficient for HSF to form a trimeric structure (59). Purified *Drosophila* HSF from heat-shocked cells is found as a trimer (60) although a hexameric form of recombinant *Drosophila* HSF has also been described (63). The COOH terminus, although less conserved in sequence, contains the transcription activation domain which was identified by fusion of segments of yeast HSF to heterologous DNA binding domains (57, 75, 76). COOH-terminal deletion mutants have revealed another interesting feature, which is that separate domains are required for transient *versus* sustained heat shock transcriptional activation (75, 76). Comparisons of *S. cerevisiae* and *K. lactis* HSF amino acid sequences and the analysis of mutant HSF have identified regions in the amino and carboxyl termini, which maintain HSF in the transcriptionally inactive state (57). Additional studies in *S. cerevisiae* have identified sequences containing repressor activity (75). The mechanism by which HSF acquires inducible transcriptional activity is not understood.

The discovery of multiple HSFs has prompted the question of their role in the stress response. Using polyclonal antisera, which specifically recognize HSF1 or HSF2, it has been shown that HSF1 is the activator of heat shock gene transcription in response to elevated temperature, heavy metals, and amino acid analogues and that HSF2 does not appear to be activated by these inducers.[2] HSF1 displays several properties consistent with this role, including stress-induced DNA binding activity, oligomerization, and translocation to the nucleus, while HSF2 does not. These findings are consistent with previous results showing that mouse HSF1 translated *in vitro* in a rabbit reticulocyte lysate acquires DNA binding activity when heated (74). In addition, the sequence of peptides obtained from HSF purified from heat-shocked HeLa cells coincided predominantly with the human HSF1 protein sequence (72, 73).

What is the function of HSF2? One answer to this question has come from studies on another inducer of heat shock gene expression. HSF2 DNA binding activity and hsp70 gene transcription are induced during hemin-induced differentiation of human K562 erythroleukemia cells (77, 78). These results suggest that HSF2 may function to activate heat shock gene expression in the absence of physiological stress, perhaps during differentiation or development. This offers possible explanations for findings of developmentally regulated expression of heat shock genes during mouse embryogenesis, avian erythoid differentiation, differentiation of the mouse male germ line, and elevated levels of constitutive HSE binding activity in unstressed embryonal carcinoma cells (79–89).

Many questions remain to be answered regarding the functional properties of HSF1 and HSF2. For example, although both HSF1 and HSF2 are able to activate hsp70 gene transcription under different conditions, it is unknown whether both factors activate transcription from all promoters with equal efficiency or whether each displays a preference for certain promoters over others. Comparison of the *in vitro* DNA binding properties and *in vivo* transcriptional activities of HSF1 and HSF2 indicates that for a given amount of DNA binding activity, HSF1 appears to be a more potent transactivator of hsp70 gene transcription than HSF2 (78). Finally, the presence of multiple HSFs in a single species creates the potential for complexes between homologous and heterologous HSF molecules. Regulation of factor activity by formation of homo- and hetero-oligomers has been observed for a number of other transcription factor families including the fos/jun, ATF/CREB, and E12, E47/MyoD families (90). In a similar fashion, modulation of the stoichiometry of potential HSF heteromultimers might alter their function and may provide cells with the ability to regulate HSF function to suit their individual needs. This is of particular interest since HSF1 and HSF2 message and protein in mouse cells and HSF-1, -2, and -3 message in chicken tissues and cell lines are co-expressed.[2,3] The presence of multiple HSFs also offers the potential for regulation not only for the classical heat shock genes but also other genes that contain HSEs, including vitellogenin, heme oxygenase, thrombospondin, IL-7, and TNF-β among others (91–96). Studies in *Drosophila* using antibodies to HSF have identified other potential targets in chromatin in addition to the traditional heat shock gene loci (58).

A Role for Heat Shock Proteins in Regulation of the Heat Shock Response

The mechanism by which cells sense elevated temperature or other forms of stress and transduce this information to the genome through the activation of HSF remains one of the critical questions in understanding the heat shock response. In the absence of stress, HSF is expressed in cells of higher eukaryotes in an inactive, non-DNA binding form and is converted to the DNA binding, transcriptionally active form by elevated temperatures

[2] K. D. Sarge, S. P. Murphy, and R. I. Morimoto, submitted for publication.
[3] A. Nakai and R. Morimoto, manuscript in preparation.

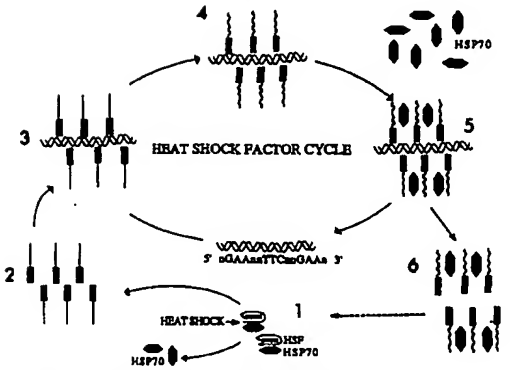

FIG 2. **The heat shock factor cycle.** A proposal for a model of HSF regulation. *1*, in the unstressed cell, HSF is maintained in a non-DNA binding state through interactions with hsp70; *2*, heat shock and other forms of stress activate HSF to an oligomeric state; *3*, HSF binds to HSEs in heat shock gene promoters; *4*, HSF can acquire post-translational modifications by phosphorylation; *5*, the levels of hsp70 increase, which leads to the formation of a HSF-hsp70 complex; *6*, dissociation of HSF from the DNA and eventual conversion to the control non-DNA binding form of HSF.

and other stresses. The failure of HSF to bind DNA is not an intrinsic property of the protein since *Drosophila* HSF and human and mouse HSF1 expressed in *E. coli* bind to DNA constitutively (63, 73).[2] In contrast, *Drosophila* HSF expressed in *Xenopus* oocytes, and mouse HSF1 and chicken HSF1 expressed in rabbit reticulocyte lysates are regulated with respect to DNA binding activity (63, 74).[3] To account for this discrepancy it has been proposed that eukaryotic cells may contain a regulatory protein that controls HSF DNA binding ability (63).

It has been speculated that the heat shock proteins may participate in the negative regulation of heat shock gene expression via an autoregulatory loop (19, 97). Experiments in *Drosophila* revealed that underexpression of hsp70 results in extended synthesis of hsp70 and overexpression of other heat shock proteins (98). Similarly, in *S. cerevisiae* mutations in hsp70 result in overexpression of the heat shock gene ssa3 which is mediated through an HSE (99). This result suggests that hsp70 is the negative regulator and that the target for this negative regulation is HSF. Additional support for the autoregulatory hypothesis comes from experimental evidence that an increase in the levels of denatured, unfolded, and mis- or malfolded proteins initiates the heat shock response. In *E. coli* and animal cells the heat shock response is induced by overexpression of mutant proteins by microinjection of denatured proteins, by treatment with amino acid analogues, and upon treatment with anti-cancer drugs that cause protein modification (100–108). Likewise, activation of the heat shock response by intermediate elevated temperatures is blocked by incubation with protein synthesis inhibitors, which suggests that damage to nascent polypeptides leads to activation of the heat shock transcriptional response (109, 112). Thus, as the levels of protein substrates for hsp70 increase, the amount of free hsp70 is depleted, relieving repression and resulting in increased expression of heat shock genes.

Direct evidence to support a regulatory role for hsp70 in HSF activation was recently shown in an *in vitro* system. Inactive HSF present in cytoplasmic extracts from non-heat-shocked HeLa cells can be activated to bind DNA upon exposure of the extract to heat, non-ionic detergents, or low pH (67, 110). Addition of hsp70 blocks the *in vitro* activation of HSF in that system (111). The inhibitory effect of hsp70 on HSF activation is relieved by addition of ATP suggesting that the inhibition is achieved by interaction of hsp70 with HSF since ATP hydrolysis is required for release of hsp70 from substrate. However, a direct demonstration of hsp70 interaction with inactive HSF remains to be shown. Studies using anti-hsp70 antibodies in conjunction with the gel shift assay demonstrate that hsp70 is found in a complex with heat-activated HSF (111, 112). Association of hsp70 with activated HSF does not hinder the DNA binding ability of HSF. It remains to be determined whether the association of hsp70 modulates the transcriptional activity of HSF.

A model for the regulation of HSF is shown in Fig. 2. Under non-stressful conditions of cell growth HSF is maintained in a non-DNA binding form through interactions with hsp70. This association, which need not be as a stable complex, maintains HSF in its non-DNA binding state, possibly by stabilizing the folded conformational state of control HSF. During heat shock, the appearance of denatured, misfolded, and malfolded proteins creates a large pool of new protein substrates, which compete with HSF for association with hsp70. Thus, heat shock and other stresses initiate the events that remove the negative regulatory influence on HSF DNA binding activity. The released HSF oligomerizes, binds DNA, and acquires transcriptional activity. The activation of HSF DNA binding leads to the elevated transcription, synthesis, and accumulation of heat shock proteins, which then associate with HSF. The association of HSF with hsp70 may be important in the regulation of its transcriptional activity and/or conversion back to the control form.

Reconstitution of Regulated Heat Shock Transcription

A complete understanding of heat shock transcription will involve the reconstitution *in vitro* of the purified components. Early studies on the chromatin structure of *Drosophila* and yeast heat shock genes indicated that binding of HSF is inducibly regulated while the TATA factor(s) are constitutively bound. Studies on the DNase I sensitivity of the upstream regions of *Drosophila* heat shock genes indicated the absence of nucleosomes (48, 52, 113–117). Recently, it was shown from *in vitro* studies that HSF, unlike the transcription factor GAL4, does not bind to nucleosome-containing templates unless the TATA factor TFIID is present (118, 119). Additionally, purified HSF and recombinant HSF are active in *in vitro* transcription extracts on HSE-containing DNA templates; however, the -fold activation does not reach the level of *in vivo* induction (50, 62, 71). Thus the role of chromatin structure and interactions of HSF with other components of the transcriptional machinery are likely to be important for proper regulation.

Regulation of heat shock gene transcription may be more readily addressed in *Drosophila* as the promoters are relatively simple, only one HSF is present, and it has already been demonstrated that RNA polymerase II is associated with each hsp70 promoter prior to transcriptional induction by heat shock (120). Each molecule of RNA polymerase is engaged and has synthesized a short transcript of approximately 25 nucleotides (64). Following heat shock, this block in elongation is lifted leading to rapid transcriptional induction. By comparison with the situation in *Drosophila*, the vertebrate heat shock promoters are more complex and there are multiple HSFs. These features provide additional complexities that will continue to reveal new insights to the understanding of heat shock gene regulation.

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